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Performance characteristics and estimation of measurement uncertainty of three plating procedures for *Campylobacter* enumeration in chicken meat

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Abstract

In this work, we present an intra-laboratory study in order to estimate repeatability (r), reproducibility (R), and measurement uncertainty (U) associated with three media for *Campylobacter* enumeration, named, modified charcoal cefoperazone deoxycholate agar (mCCDA); Karmali agar; and CampyFood ID agar (CFA) a medium by Biomérieux® SA. The study was performed at three levels: (1) pure bacterial cultures, using three *Campylobacter* strains; (2) artificially contaminated samples from three chicken meat matrixes (total $n = 30$), whereby samples were spiked using two contamination levels; ca. 10^3 cfu *Campylobacter*/g, and ca. 10^4 cfu *Campylobacter*/g; and (3) pilot testing in naturally contaminated chicken meat samples ($n = 20$).

Results from pure culture experiment revealed that enumeration of *Campylobacter* colonies on Karmali and CFA media was more convenient in comparison with mCCDA using spread and spiral plating techniques. Based on artificially contaminated samples testing, values of repeatability (r) were comparable between the three media, and estimated as 0.15 $\log_{10}$ cfu/g for mCCDA, 0.14 $\log_{10}$ cfu/g for Karmali, and 0.18 $\log_{10}$ cfu/g for CFA. As well, reproducibility performance of the three plating media was comparable. General R values which can be used when testing chicken meat samples are; 0.28 $\log_{10}$, 0.32 $\log_{10}$, and 0.25 $\log_{10}$ for plating on mCCDA, Karmali agar, and CFA, respectively. Measurement uncertainty associated with mCCDA, Karmali agar, and CFA using spread plating, for combination of all meat matrixes, were $\pm$0.24 $\log_{10}$ cfu/g, $\pm$0.28 $\log_{10}$ cfu/g, and $\pm$0.22 $\log_{10}$ cfu/g, respectively. Higher uncertainty was associated with Karmali agar for *Campylobacter* enumeration in artificially inoculated minced meat ($\pm$0.48 $\log_{10}$ cfu/g).

The general performance of CFA medium was comparable with mCCDA performance at the level of artificially contaminated samples. However, when tested at naturally contaminated samples, non-*Campylobacter* colonies gave similar deep red colour as that given by the typical *Campylobacter* growth on CFA. Such colonies were not easily distinguishable by naked eye.

In general, the overall reproducibility, repeatability, and measurement uncertainty estimated by our study indicate that there are no major problems with the precision of the International Organization for Standardization (ISO) 10272-2:2006 protocol for *Campylobacter* enumeration using mCCDA medium.

Keywords: *Campylobacter* enumeration; Repeatability; Reproducibility; Measurement uncertainty

1. Introduction

Campylobacter jejuni and *Campylobacter coli* are now recognised as significant causes of foodborne bacterial disease worldwide. Consumption or mishandling of raw or undercooked poultry meat products has been identified as major risk for human infection (Butzler, 2004; Snelling et al., 2005). The 2005 Community Summary Report on Trends of Zoonoses in the European Union highlighted campylobacteriosis as the most frequently reported zoonotic disease in humans within the EU. Reported cases

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increased by 7.8% compared to previous year, rising to an average annual incidence of 51.6 cases per 100,000 people and to a total of 197,363 recorded cases (Anonymous, 2006d). The likelihood of human illness with *Campylobacter* follows a dose–response relation in which the initial concentration of *Campylobacter* in contaminated food plays an important role (Teunis et al., 2005). Only as few as 500 bacteria can induce clinical symptoms (Black et al., 1988).

The need for quantitative data on the concentration of *Campylobacter* in chicken meat has increased dramatically since the onset of quantitative microbial risk assessment. However, the availability of such quantitative data is a challenging issue in some countries (Uyttendaele et al., 2006). In response to the requirement for a robust method for the enumeration of *Campylobacter* in foods and related matrixes, the International Organization for Standardization (ISO) has issued, in the year 2006, a quantitative protocol based on inoculation of the solid selective medium modified charcoal cefoperazone deoxycholate agar (mCCDA) (Anonymous, 2006b). On other hand, the French diagnostic company Biomérieux® released to the market, early 2006, a ready to use medium in Petri dishes named CampyFood ID agar (CFA), claiming the advantage of selective isolation of *Campylobacter* in food, as well as the ease of identifying presumptive colonies due to the presence of colouring indicator included in medium ingredients (Anonymous, 2006a). However, while considering valid microbiological methods, such methods should be shown to be fit for their intended purpose. Therefore, performance testing for the new standard method and alternative plating methods is required to show the fit of such methods for a realistic *Campylobacter* enumeration performance across the full range of the foods to be tested, taking into account the effect of different levels of the target organisms.

Whilst for many years, food microbiologists have estimated the reliability of their quantitative enumeration methods at best as $\pm 0.5 \log_{10}$ cfu, the task now is to refine such statement to provide reliable and objective estimate for uncertainty of results obtained by microbiological methods (Corry et al., 2007). Uncertainty of measurement is a quantitative indication of the analytical variability of a result. It demonstrates how well the result represents the value of the quantity being measured in the test portion. International consensus supports the use of reproducibility (R) and repeatability (r) data in the evaluation of uncertainty in food microbiology (Lombard, 2006; Corry et al., 2007). The precision of a measurement which is the closeness of agreement between independent test results can be expressed as (a) the repeatability which is the precision under repeatability conditions (independent results obtained on identical test items with the same method, in the same laboratory, by the same operator, using the same equipment, and within short interval of time), and (b) the reproducibility which is the precision under reproducibility conditions (results obtained on identical test items with the same method under different conditions (e.g. operators and equipments)) (Augustin and Carlier, 2006). Reproducibility data can be generated using inter-laboratory study, inter-laboratory proficiency trial, and intra-laboratory study. Using data based on the intra-laboratory standard deviation of reproducibility (S_R) is the first option for estimation of measurement uncertainty (U) associated with enumeration microbiological methods (Lombard, 2006).

We aimed in the present work to conduct an intra-laboratory study to measure the repeatability (r), reproducibility (R), and measurement uncertainty (U) associated with three plating media, namely mCCDA, Karmali agar, and CFA. Results were generated using a carefully designed artificial contamination protocol, targeting a range of three broiler meat matrixes representative of those routinely analysed in food microbiology laboratories.

2. Material and methods

2.1. Campylobacter strains

Three *Campylobacter* strains from poultry origin were used in the study: (i) *C. jejuni* LFMFP 591; (ii) *C. jejuni* LFMFP 592; and (iii) *C. coli* LHT 595. The first two were provided from the culture collection of the Laboratory of Food Microbiology and Food Preservation, Faculty of Bioscience Engineering, and the third from the Laboratory of Hygiene and Technology, Faculty of Veterinary Medicine, both at Ghent University. A reference stock of each strain was stored at −80 °C in full-horse blood (E&O laboratories, England). A stock culture was prepared by suspending a sterile swab from the reference stock in 10 ml of Bolton broth (BB, CM983 with supplement SR183, Oxoid, England) and incubated for 24 h at 37 °C in a microaerobic atmosphere (in anaerobic jar using Campygen CN0025, Oxoid, England). After incubation, stock cultures were kept at 4 °C in BB under microaerobic conditions, and renewed on monthly basis. A working culture was prepared by transferring 0.1 ml of the stock culture into 10 ml of fresh BB, and incubated for 24 h at 37 °C to reach a stationary phase culture ($\sim 10^7$–10^8 cfu/ml). Purity and verification of the *Campylobacter* cultures concentration were confirmed by 10-fold serial dilutions from working culture into peptone water (PW) and spread plating 0.1 ml from selected dilutions onto duplicates of mCCDA (CM739 and supplement SR155, Oxoid, England), and duplicates of Muller–Hinton based blood agar plates (MH, CM337 Oxoid, England; and supplemented with 5% (v/v) full-horse blood, E&O laboratories, England).

2.2. Pure culture experiment

A working culture from each of the three strains was kept on BB as described before (Section 2.1). This freshly prepared working culture was identified in the course of the experiment as day zero working culture (D0 WC), part of it

used immediately for serial dilution and plating, while the rest was kept in laboratory refrigerator at 5 °C under aerobic condition for 72 h, achieving what was identified as day 3 working culture (D3 WC). The aim of this conservation step was to mimic cold storage of meat and to induce a stress factor over *Campylobacter* working cultures through aerobic storage.

Each of two laboratory analysts working in parallel prepared two sets of 10-fold serial dilutions from D0 WC, and D3 WC of each of the three *Campylobacter* strains. Thus, six replicates experiments for each analyst. The two analysts inoculated, each in parallel using their own prepared serial dilutions set, the surface of duplicate plates of mCCDA, Karmali agar (CM935 and supplement SR167, Oxoid, England), CFA (Biomérieux® SA, France; ready-to-use in Petri dishes) and Muller–Hinton based blood agar, by aseptically transferring 0.1 ml from dilutions 10^{-4}, 10^{-5}, 10^{-6}, and 10^{-7} and spread it to dryness. Also, suspensions of 10^{-4} and 10^{-5} dilutions, from both analysts serial dilution sets, were plated using mechanical spiral platter (Eddy Jet, IUL instruments, Spain) for spiral spreading over surface of duplicates of mCCDA, Karmali agar, CFA, and Muller–Hinton based blood agar media. Agar plates were incubated for 48 h at 41.5 °C under microaerobic conditions by evacuating 80% of the normal atmosphere and introducing a gas mixture consisting of 8% CO_2, 8% H_2, and 84% N_2 into each microaerobic jar (Don Whitley Scientific, UK). After incubation, *Campylobacter* count was calculated.

2.3. Artificially contaminated samples experiment

2.3.1. Broiler meat samples

Thirty samples, representing three meat matrixes, were used: (i) minced chicken meat (10 samples: 8 chicken sausages and 2 chicken burgers); (ii) chicken leg with skin (10 samples); and (iii) skinless chicken breast fillet (10 samples). Samples were purchased from local butchers and supermarkets, stored in laboratory freezer (−21 °C) and used within two weeks. The freezing step before artificial contamination was on purpose, in order to initiate extreme physical stress conditions capable of declining, if any present, inherent *Campylobacter* in inoculated samples.

2.3.2. Experimental inoculation of meat samples

Day three working cultures (D3 WC) of *C. jejuni* LFMFP 592, and *C. coli* LHT 595 strains were used to artificially contaminate broiler meat samples. Inoculums were prepared for *C. jejuni* and *C. coli* strains at two target values; the first addressed as medium level (ca. 10^3 cfu/g), and the second as high level (ca. 10^4 cfu/g). Verification of the inoculum concentration was performed, in parallel to each artificial inoculation experiment, by 20-fold serial dilution (in PW) from the *Campylobacter* culture suspension used for sample inoculation, and spread plating 0.1 ml onto duplicate of mCCDA and duplicate of Muller–Hinton based blood agar.

For artificial contamination, a representative 10 g of each sample was homogenized with 90 ml of PW in sterile stomacher bag with filter. A negative control was taken from the initial homogenate before inoculation, and 0.1 ml from it streaked in duplicate onto mCCDA to confirm that none of the meat samples contained *Campylobacter*. The uninoculated initial homogenate was diluted to 10^{-2} and 10^{-3} suspensions, and background flora of the tested samples were evaluated by spiral plating from each dilution over duplicate of Plate Count Agar (PCA, CM0463 Oxoid, England), and incubation at 30 °C for 48 h for total aerobic bacterial count; as well as, over duplicate of RAPID's *Escherichia coli* medium (Bio-Rad, France), and incubation at 44 °C for 18 h for total *E. coli* count.

Calibrated quantity of *Campylobacter* cultures representative to desired inoculum levels (spiking procedures presented in Table 1) were aseptically transferred to the stomacher bag containing the sample homogenate and mixed for another 1 min to achieve a uniform distribution of *Campylobacter* inoculum. Inoculated samples were analysed immediately after artificial contamination.

2.3.3. Intra-laboratory estimation of plating media reproducibility (*R*) and repeatability (*r*)

Meat sample (total 30) of three chicken meat matrixes were artificially contaminated as described before. For estimation of reproducibility (*R*) performance of media under evaluation, the initial sample homogenate (10^{-1}) and a following dilution in PW (10^{-2}) were prepared according to the desired *Campylobacter* inoculum levels (Table 1). Plating was performed by two analysts in parallel; from each sample, each analyst used the same primary dilution (10^{-1}) of inoculated test portion, and going from this, each prepared a further serial (10-fold) dilution. Media plating was carried under a pre-designed reproducibility assay that included variations as may be encountered from one routine working day to another within the laboratory: different technicians, different batches of media and reagents, different vortex mixers, pH metres, pippeting and handling equipments, and different incubators.

Plates were incubated for 48 h at 41.5 °C, under microaerobic conditions, and presumptive positive colonies were enumerated. All results of negative control (uninoculated portion) from tested samples confirmed that none of them contained *Campylobacter*. Therefore, a short confirmation of presumptive colonies to genus level was performed based on positive oxidase reaction, growth under microaerobic conditions, and microscopic determination of cell morphology using Gram-stain and examination for presence of the Gram-negative, curved shaped bacterial cells (Anonymous, 2006b).

Repeatability (*r*) performance of mCCDA, Karmali agar, and CFA media, was assessed by one analyst working under repeatability conditions (e.g. same analyst, using same mixer, pippeting, and handling equipments, same batch of media, and same incubator) and within short time interval (Anonymous, 2005a). The analyst performed five

Table 1
Distribution of *Campylobacter* inoculums for a spiked meat matrix of 10 samples, and following plating procedures used in reproducibility experiment

Inoculum level	Description	n[a]	Reproducibility experiment	
			Dilutions	Plating procedures[b]
Medium	ca. 10^3 cfu *C. jejuni*/g	2	10^{-1}	• 1 ml, spread plating over three plates, in duplicate ($3 \times 2 = 6$). • And, 0.1 ml, spread plating, in duplicate.
	ca. 10^3 cfu *C. coli*/g	2	10^{-1}	• 1 ml, spread plating over three plates, in duplicate ($3 \times 2 = 6$). • And, 0.1 ml, spread plating, in duplicate.
High	ca. 10^4 cfu *C. jejuni*/g	3	10^{-1}	• 0.1 ml, spread plating, in duplicate. • And, spiral plating, in duplicate.
			10^{-2}	• 0.1 ml, spread plating, in duplicate.
	ca. 10^4 cfu *C. coli*/g	3	10^{-1}	• 0.1 ml, spread plating, in duplicate. • And, spiral plating, in duplicate.
			10^{-2}	• 0.1 ml, spread plating, in duplicate.

[a] $n = 10$ tested samples per each matrix, the same inoculums description, and the same samples distribution were applied to the three matrixes (minced meat, chicken legs with skin, skinless chicken breast fillet).
[b] The same plating procedures were applied to the three media.

replicates of spread plating of 0.1 ml from the same initial homogenate of samples artificially inoculated with ca. 10^4 cfu *C. coli*/g (one sample tested per each of the three chicken meat matrixes). Replicates were incubated for 48 h at 41.5 °C, under microaerobic conditions, and verification of inoculum density, negative control confirmation of absence of *Campylobacter* and check over background flora were conducted as described before.

2.4. Naturally contaminated samples testing

A total of 20 samples of raw chicken meat products (16 meat preparations, and 4 chicken legs with skin) were collected from 3 meat processing plants. Samples were analysed within 3–4 h from collection. Representative 10 g portion were aseptically weighted and homogenised with 90 ml PW. Serial decimal dilutions were prepared with the same diluent. Spread plating from 10^{-1} dilution was performed using 1 ml over four plates (0.3, 0.3, 0.3, and 0.1 ml), and from 10^{-2} dilution using 0.1 ml. In all cases, this procedure was done on all three agar types under study. Incubation and confirmation of presumptive colonies were conducted as described in ISO 10272-2:2006 (Anonymous, 2006b).

2.5. Statistical analysis

All cfu results, per gram or millilitre, were transformed to $\log_{10}$ counts before statistical analysis in order to stabilise the reproducibility variance over the contamination levels (Anonymous, 2006c). Results based on estimated counts were excluded, because standard methods require that count below or above the optimal range (15–150 cfu) must be reported as estimates, and hence they could not be used in precision analysis (Anonymous, 2005b, 2006c). During any intermediate stages of calculations, data were kept as integer figures as possible and only rounded at final results calculations, to avoid rounding errors.

As distribution of cfu in Petri dishes follows a Poisson series, the significant differences (P-value $\leqslant 0.05$) between counts obtained using the three plating media and between plating procedures, as well as within meat matrixes, were tested using generalised linear models (GLMs) starting with Poisson regression analysis, and in case of extra-Poisson dispersion, the analysis was continued with a negative binomial regression analysis beginning with the general form.

The standard deviation for reproducibility (S_R) and for repeatability (S_r) were calculated, and uncertainty of measurement (U) was obtained using the reproducibility standard deviation (S_R) of $\log_{10}$ transformed data, by applying the following formula (Anonymous, 2006c):

$$U = \log_{10}(c) \pm [k \times S_R]$$

where c is the $\log_{10}$ count; S_R the reproducibility standard deviation; and k is the appropriate coverage factor (usually 2 to obtain the expanded uncertainty; below the number of 30 samples sets of paired counts the coverage factor was determined by using the Student's t-value for the number (n) of sample sets and the 0.975 quantile (two-tailed 95% test) with $n-1$ degrees of freedom).

Agreement between count results obtained at pure culture testing from spread and spiral plating were assessed

Table 2
Descriptive statistics for *Campylobacter* pure culture experiment

Plating methods	Media	Total[a] countable results	Paired[b] countable results	[c]Count based on condition-operator A		[c]Count based on condition-operator B	
				Mean ± SD	Min–Max	Mean ± SD	Min–Max
Spread	mCCDA	3/12	0/6	–	–	–	–
	Karmali	12/12	6/6	7.49 ± 0.46	6.92–8.09	7.19 ± 0.58	6.35–7.79
	CFA	12/12	6/6	7.59 ± 0.53	6.96–8.29	7.09 ± 0.85	5.81–7.88
Spiral	mCCDA	3/12	2/6	–	–	–	–
	Karmali	12/12	6/6	7.63 ± 0.45	6.95–8.07	7.78 ± 0.55	6.93–8.37
	CFA	12/12	6/6	7.86 ± 0.57	7.11–8.56	7.95 ± 0.58	7.25–8.63

[a]Total experiments = 12, calculated as, 3 *Campylobacter* strains × 2 culture ages per each strain, tested in parallel by two analysts.
[b]Paired countable results obtained in parallel from the two analysts working under independent conditions (condition A and B).
[c]Counts presented as mean $\log_{10}$ cfu/ml ± standard deviation, and minimum and maximum counts.

using two statistical methods. A concordance correlation coefficient (*CCC*) was calculated to determine the overall level of agreement between *Campylobacter* enumeration results by measuring the variation in counts linearity from 45° line through the origin. Therefore, this coefficient is not only measuring how far each count deviates from the line fit to the data (precision of result), but also how far this line deviated from the 45° line of origin (accuracy of result) (Lin, 1989). The second statistical method was Bland–Altman plot, where the limits of agreement are visually presented to show the difference between pairs of test results relative to their mean value. Lines that denote the upper and lower difference values that enclose 95% of the points are added to the plot (Bland and Altman, 1995). All statistical analyses were performed using computer program STATA for windows, version 8 (StataCorp, 2003).

3. Results

3.1. Media performance using Campylobacter pure cultures

Table 2 summarizes the results of the pure cultures experiment. At this level of media evaluation, mCCDA results were the least countable among the three evaluated media. No paired counts were generated from spread plating on mCCDA due to partial or total swarming of bacteria which made counting impossible or doubtful. In view of the few generated data from mCCDA medium at the level of pure culture experiments, descriptive statistics for its enumeration results were foreseen to be of little value at this point of the study.

On other hand, shapes of colonies on Karmali agar and CFA were, generally, well circumscribed and enumeration was successful using spiral and spread plating techniques. GLM starting with Poisson regression analysis indicated no significant difference between counts obtained on Karmali agar using spread and spiral plating ($P = 0.747$), and so on CFA using spread and spiral plating ($P = 0.620$). However, when the model aimed at estimating the effect of plating techniques on *Campylobacter* counts, a negative binomial regression analysis indicated that counts obtained using spiral plating technique were significantly higher ($P = 0.002$) than those obtained in parallel using spread plating technique, that was regardless the type of medium (Karmali or CFA) incorporate in plating procedures (Fig. 1).

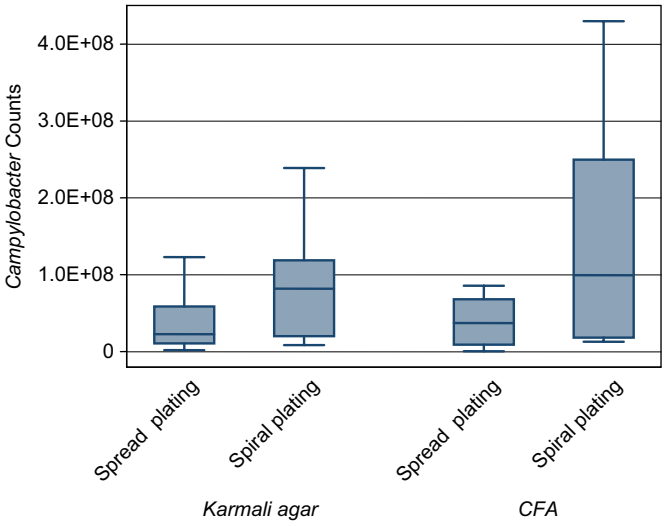

Fig. 1. Box plot comparing *Campylobacter* counts, obtained from pure culture experiment, using spiral versus spread plating techniques on Karmali and CFA media.

When counts obtained using spread plating on CFA were plotted against those of Karmali agar, the *CCC* was 0.840 (95% confidence interval = 0.692, 0.989). The bias correction factor (*BCF*) measured how far the best-fit deviated from the 45° line (measure of accuracy), was measured at 0.949, which showed that line of best-fit was very close to the perfect agreement line. On other hand, the *CCC* between Karmali and CFA for *Campylobacter* counts obtained using spiral plating was 0.844 (95% confidence interval = 0.689, 1.000). And *BCF* was 0.920, which showed that line of best-fit was very close to the perfect line.

Using Bland–Altman method of assessing agreement (Fig. 2A), the 95% limits of agreement between results

from both media were −0.706 and 0.705 $\log_{10}$ cfu, respectively, indicating that 95% of pairs of *Campylobacter* enumeration results differed by less than, approximately, ±0.7 $\log_{10}$ cfu. While, 95% limits of agreement between results from both media using spiral plating was −0.633 and 0.236 $\log_{10}$ cfu, respectively. That was relatively better than agreement limits based on using spread plating with 95% of pairs of *Campylobacter* enumeration results on Karmali and CFA differed by less than, approximately, ±0.45 $\log_{10}$ cfu (Fig. 2B).

3.2. Media performance using artificially contaminated matrixes

Table 3 shows mean counts obtained per *Campylobacter* inoculum levels, precision parameters, and measurement uncertainty for each artificially contaminated combination (plating method, meat matrix, and medium). Parameter values were calculated according to ISO/TS 19036:2006 (Anonymous, 2006c). The standard deviation of reproducibility (S_R) was estimated for the group of *Campylobacter* strains as a consistent group of target microorganisms, for each medium and for each inoculated matrix, and for combined results from all meat matrixes. It is difficult to recommend exactly how many pairs of counts are required to have a reasonably precise estimate of the average S_R without additional information. However, review of technical standards indicates that, ideally, at least 30 sets of paired counts (paired counts = count set obtained from two analysts working in parallel under reproducibility conditions) should be used, but a reasonable estimate may be obtained using 10 sets of paired counts (Anonymous, 2005b, 2006c). Therefore, we aimed in our study to generate 10 sets of paired counts per each of the three artificially inoculated matrixes, achieving a total of 30 sets of paired counts (Table 3).

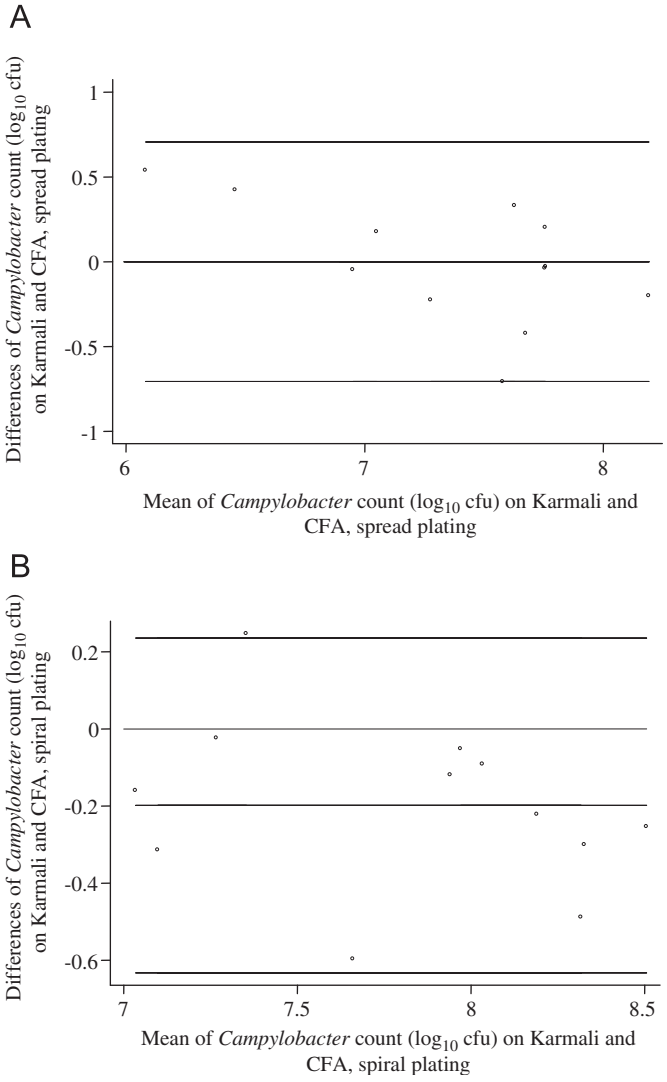

Fig. 2. Bland–Altman plot showing 95% limits of agreement between *Campylobacter* counts obtained using Karmali and CFA media in pure cultures experiment. Difference between counts is plotted against their mean value, with upper and lower horizontal lines showing the 95% limits of agreement. (A) Using spread plating technique and (B) using spiral plating technique.

For spiral plating, there were no paired sets of counts obtained for mCCDA. And, there was no significant difference between performance parameters calculated for *Campylobacter* enumeration on Karmali agar and CFA using spiral platting (Table 3). For spread plating, the least numbers of sets of paired counts were associated with Karmali agar results for testing artificially inoculated minced meat. Minced meat samples contained aerobic plate count of ca. 10^5 cfu/g, and *E. coli* count of ca. 10^3 cfu/g. Such relatively high level of background flora might be the cause of failure of enumeration on some of Karmali plates, especially, when plates received volumes of 0.3 and 0.4 ml (data not presented). Growth of contaminants was less with mCCDA plates inoculated in parallel with the same volumes. Counting on CFA was not compromised by the relatively high load of inherent flora in minced meat samples (Table 3).

The three evaluated media showed a comparable repeatable performance. For the ISO 10272-2:2006 enumeration protocol using mCCDA, a general value of $r = 0.15 \log_{10}$ can be used when examining poultry meat samples. For plating on Karmali and CFA media, the estimated r values were 0.14 $\log_{10}$ and 0.18 $\log_{10}$, respectively. Values of repeatability within each of the three matrixes are presented in Table 3. As well, reproducibility performance of the three plating media was comparable. Global R values which can be used when examining poultry meat samples are 0.28 $\log_{10}$, 0.32 $\log_{10}$, and 0.25 $\log_{10}$ for plating on mCCDA, Karmali, and CFA, respectively (Table 3).

For all meat matrixes, mCCDA and CFA media were associated with a comparable measurement uncertainty, estimated as ±0.24 $\log_{10}$ cfu/g and ±0.22 $\log_{10}$ cfu/g, respectively (Table 3). On other hand, the highest estimated uncertainty (±0.48 $\log_{10}$ cfu/g) was associated with Karmali counts in minced meat preparations.

Table 3
Performance parameters of three plating procedures for enumeration of *Campylobacter* on three artificially contaminated chicken meat matrixes

Plating method	Chicken meat matrixes	Media	Performance characteristics					Mean count ($\log_{10}$ cfu/g)	
			No. of paired counts sets	[a]S_R ($\log_{10}$)	[b]R ($\log_{10}$)	[c]U ($\pm\log_{10}$)	[d]S_r ($\log_{10}$)	[e]r ($\log_{10}$)	Medium inoculum	High inoculum
Spiral	All meat matrixes	Karmali	14/18	0.055	0.126	0.121				
		CFA	17/18	0.071	0.163	0.151				
Spread	All meat matrixes	mCCDA	29/30	0.123	0.280	0.245	0.066	0.152	3.37	4.07
		Karmali	27/30	0.139	0.318	0.286[A]	0.063	0.144	3.36	4.16
		CFA	30/30	0.110	0.250	0.220	0.081	0.185	3.39	4.11
	Minced meat	mCCDA	9/10	0.141	0.322	0.325	0.052	0.120	3.48	3.93
		Karmali	7/10	0.195	0.445	0.478[A]	0.068	0.155	3.39	4.07
		CFA	10/10	0.112	0.256	0.254[B]	0.066	0.150	3.38	4.09
	Chicken leg with skin	mCCDA	10/10	0.074	0.169	0.168	0.104	0.236	3.30	4.14
		Karmali	10/10	0.100	0.229	0.227[A]	0.085	0.193	3.33	4.20
		CFA	10/10	0.107	0.244	0.242[A]	0.095	0.217	3.34	4.06
	Chicken breast fillet	mCCDA	10/10	0.142	0.325	0.322	0.043	0.098	3.34	4.17
		Karmali	10/10	0.126	0.286	0.284[B]	0.037	0.084	3.36	4.22
		CFA	10/10	0.110	0.252	0.250[B]	0.081	0.184	3.44	4.18

[a](S_R) Standard deviation of reproducibility: is the standard deviation of test results obtained under reproducibility conditions.
[b](R) Reproducibility limit: is the value less than or equal to which the absolute difference between two test results obtained under reproducibility conditions is expected to be with probability of 95%, $R = 2.28 \times S_R$.
[c](U) Uncertainty of measurement: parameter, associated with the result of a measurement, that characterizes the dispersion of the values that could reasonably be attributed to the measurand.
[d](S_r) Standard deviation of repeatability: is the standard deviation of test results obtained under repeatability conditions.
[e](r) repeatability limit: is the value less than or equal to which the absolute difference between two test results obtained under repeatability conditions is expected to be with probability of 95%, $r = 2.28 \times S_r$.
[A]Indicates a significantly higher difference ($P \leqslant 0.05$).
[B]Indicate a significantly lower difference ($P \leqslant 0.05$).

3.3. Pilot testing of media performance in naturally contaminated samples

Table 4 shows that plating using mCCDA medium was associated with higher number of total countable results in comparison with results obtained using CFA and Karmali agar media. Samples which shown to be negative on mCCDA were also negative for plating on Karmali and CFA media.

On other hand, non-*Campylobacter* growth on surface of the three media were also identified by their atypical colony morphology and Gram-staining. These colonies were relatively easily distinguishable from the typical *Campylobacter* growth on mCCDA, but were more difficult to identify on Karmali agar. As shown in Table 4, CFA medium was associated with the highest level of non-*Campylobacter* contaminants. Such confusing colonies were not easy identifiable by naked eye, but we were able to exclude them by using stereomicroscope with Henry illumination to get details over colony morphology. Distinguishing the atypical *Campylobacter* colonies from the typical ones was also achievable by colonies purification on Muller–Hinton based blood agar, and microaerobic incubation at 41.5 °C for 24 h. Growth on blood agar indicated that majority of non-*Campylobacter* contaminants grown on CFA were of haemolytic nature and by Gram-staining they showed to be Gram-positive long bacilli or cocco-bacilli. That was enough to exclude them from being enumerated as *Campylobacter*, however, we did not completely identify those atypical colonies to genus level due to level of work required in this study.

Table 4
Media performance using naturally contaminated chicken meat samples

	mCCDA	Karmali	CFA
Countable results/total tested samples	16/20	11/20	12/20
Confirmed *Campylobacter* spp. count Mean $\log_{10}$ per gram $\pm$ SD	2.24 $\pm$ 0.51	2.60 $\pm$ 0.33	2.56 $\pm$ 0.45
Non-*Campylobacter* contaminants count Mean $\log_{10}$ per gram $\pm$ SD	2.15 $\pm$ 0.29	2.53 $\pm$ 0.63	3.62 $\pm$ 0.33

4. Discussion

While *Campylobacter* is heading to the top of the most frequently occurring zoonotic diseases in Europe, there is a need for realistic quantitative risk assessment approach based on reliable quantitative data (Rosenquist et al., 2003;

Hutchison et al., 2006). The present study provides an intra-laboratory evaluation of the performance characteristics, and measurement uncertainty associated with three plating media; the ISO 10272-2:2006 recommended enumeration medium, mCCDA; Karmali agar, a widely regarded medium for selective isolation of *Campylobacter* with detection performance most comparable to mCCDA (Jacobs-Reitsma and de Boer, 2001); as well as, CFA, a recently released selective medium, by Biomérieux® SA France, for isolation of *Campylobacter* from food samples. There are few published comparisons to support evaluation of quantitative *Campylobacter* detection methods, as most of published studies focus on qualitative detection (presence/absence) (Corry et al., 1995; Wang, 2002; Tangvatcharin et al., 2005).

In the first part of the study, we presented a comparison between plating media and plating techniques for their enumeration performance using pure cultures of three *Campylobacter* strains. mCCDA and Karmali agar are examples of *Campylobacter* plating media that include charcoal instead of blood (Corry et al., 1995). Jacobs-Reitsma and de Boer (2001) reported that mCCDA and Karmali agar are essentially similar in sensitivity and productivity if the aim of testing is detection of *Campylobacter*. Gun-Munro et al. (1987) compared counts on Skirrow, Butzler, Blaser-Wang, Preston, mCCDA, and Karmali media using pure cultures of 70 strains of *C. jejuni*. All media except Skirrow, mCCDA, and Karmali gave slightly but significantly lower mean counts than non-selective blood agar. And, when inoculation was extended to simulated faeces samples their study concluded that *Campylobacter* colonies were easier to be recognised on Karmali agar than on mCCDA. The previous conclusion is matching with what we experienced at pure culture level experiment, as counting was easily achieved on Karmali agar, and CFA medium as well, while swarming of *Campylobacter* on mCCDA surface made generating precise counts a difficult task using pure cultures.

In order to evaluate agreement between *Campylobacter* counts obtained using Karmali agar and CFA media, we used the *CCC* and Bland–Altman plot (Lin, 1989; Bland and Altman, 1995). These statistical tools become very popular in analytical chemistry and quantitative comparisons of medical diagnostics. In our study, we described an example for their applicability in the field of food microbiology. Results at pure cultures level revealed high degree of accuracy, precision, and high level of agreement between both media. Also, the use of mechanical spiral plating shown to be a reliable technique, where 95% of pairs of *Campylobacter* enumeration results differed by less than $\pm 0.45 \log_{10}$ cfu compared to $\pm 0.7 \log_{10}$ cfu for parallel results obtained using spread plating (Fig. 2). Considering the fastidious and microaerobic nature of *Campylobacter*, the use of spiral plating technique provides advantages of fast spreading, efficient dryness, and minimise unnecessary contact with air. However, based on pure culture testing (Table 2), the choice of *Campylobacter* isolation medium was an important factor for successful generation of countable results. We experienced that swarming of bacteria on mCCDA led to continuous, uncountable, circular chains of bacterial growth as a result of interaction between plated spiral lines. Whereas on Karmali and CFA media, colonies were generally well defined and counting was possible using spiral plating technique, even when plating high concentration of *Campylobacter* cultures (ca. 10^4 cfu/ml).

The finding that *Campylobacter* pure cultures counts obtained using spiral plating technique were higher than those obtained by spread plating technique is an interesting finding (Fig. 1). Such finding is supported by statistical evidence that this significant difference is an influence of the plating technique regardless of the type of medium used. There is no obvious explanation for this phenomenon. However, Augustin and Carlier (2006) indicated through inter-laboratory comparisons conducted within a French proficiency testing program that laboratories using spiral plater obtained systematically higher counts of aerobic microorganisms, *Enterobacteriaceae*, coliforms, and staphylococci than others using usual dilution and plating techniques.

On the other hand, the values estimated through our intra-laboratory evaluation (Table 3) can be used as a surrogate for r, R, and U values for *Campylobacter* enumeration in other poultry meat matrixes. However, the ideal use of our estimated values will be applicable, strictly, to *Campylobacter* concentrations and meat matrixes comparable to those studied.

Our parameters estimation was based on testing artificially contaminated samples, using two contamination levels (ca. 10^3 and 10^4 cfu *Campylobacter*/g). The calculation of reproducibility and repeatability standard deviation on log-transformed data stabilizes the variance over the contamination levels, and therefore there was no need to estimate standard deviation per each contamination level (Anonymous, 2006c). While designing our inoculation experiment, it was the aim to include contamination levels comparable to *Campylobacter* concentration range in raw broiler meats. Meanwhile, it was necessary that chosen contamination levels allow us to avoid results based on low counts, as the current ISO approach for estimation of measurement uncertainty in food microbiology does not cover estimation based on counts less than 10 cfu/g or ml due to the lack of a simple agreed approach (Anonymous, 2006c).

Only recently, precision parameters for a number of ISO methods were investigated. That includes enumeration methods for *Bacillus cerus*, *Listeria monocytogenes*, *Staphylococcus aureus*, and *Clostridium perfringens* (Uyttendaele and Debevere, 2006). Our estimation of performance parameters for *Campylobacter* enumeration based on the standard ISO 10272-2:2006 is lower than those obtained for the previous pathogens. However, we used an intra-laboratory approach, while validation studies for the previously mentioned pathogens were based on inter-laboratory investigations. Such studies design involves

more variables in testing conditions than ours. This should be taken into consideration when comparing our study with future studies concluding precision parameters values for *Campylobacter* enumeration methods. On the other hand, the estimation of measurement uncertainty (U) based on intra-laboratory S_R, as the case in our study design, is a preferred option over estimation based on inter-laboratory S_R derived from method validation or proficiency testing. Intra-laboratory S_R provides a measurement uncertainty value that is linked to the laboratory, thus nearer to the basic measurement uncertainty definition which relates measurement uncertainty even to one individual test result (Lombard, 2006).

Knowledge of the uncertainty of bacterial counts is essential to adequately interpret these counts. Taking into account the uncertainty allows, for example, to determine if differences between results obtained on the same samples are in the acceptable range of the experimental variability, or to confirm that samples are in accordance with given specifications (Corry et al., 2007; Augustin and Carlier, 2006). Ideally each measurement should be quoted with an indication of the uncertainty, often as a $\pm$figure, so that decisions based on the measurement are fully informed (Lombard, 2006). The estimated U-value for spread plating on mCCDA was $\pm 0.24 \log_{10}$ cfu/g as a general value for combination of all meat matrixes results. On the other hand, higher uncertainty and less paired counts sets were associated with Karmali agar when tested artificially contaminated chicken matrix contained high level of background flora (Table 3). Typically, when the count of *Campylobacter* in meat preparations is expected to be low, plating a volume of 1 ml from initial sample homogenate is required to achieve reliable laboratory detection limits. We experienced that Karmali plates receiving volumes of 0.3 and 0.4 ml were more prone to be rejected as uncountable, due to overspread of film and/or clusters of background flora over colonies of presumptive *Campylobacter* growth. Previous findings should be considered when enumeration data, or even detection, are required for minced poultry meats, and grounded meat preparations like chicken sausages and burgers.

The general performance of the new chromogenic-like medium CampyFood ID (CFA) was of notable agreement with Karmali agar at level of *Campylobacter* pure cultures testing, and generally comparable with mCCDA performance using artificially contaminated samples. However, we initiated pilot testing of CFA medium at naturally contaminated samples levels (Table 4), and based on our built up practical experience with this new medium, it is worth to mention that in many cases non-*Campylobacter* colonies on CFA gave deep red (burgundy) to orange–red colour undistinguishable from that given by the typical *Campylobacter* growth. In our study, these type of colonies were more abundant when testing naturally contaminated samples and was less in artificially inoculated samples as the latter were subjected to prolonged freezing before inoculation. However, typical *Campylobacter* growth did not show any tendency to spread on CFA medium, and the ease of recording results was by far the most convenient in comparison with mCCDA and Karmali agar. The performance of CFA medium worth to be investigated in further studies, taking into account its performance for isolation of *Campylobacter* after pre-enrichment in parallel to direct plating as well.

In conclusion, our study highlights the importance of a multilevel evaluation strategy in validation of alternative quantitative methods or assessment performance of standard methods in food microbiology. In the present evaluation, accurate conclusion was only possible to be drawn by testing media performance using *Campylobacter* pure cultures, artificially inoculated, and naturally contaminated samples all together in a context and not separately. If a conclusion was set only on results of pure cultures testing, that would mislead by indicating that mCCDA performance is inferior to Karmali agar and CFA media. And if we based our conclusion only on artificially contaminated samples experiments, we would come to a premature indication that CFA medium is the best of the three compared media. Karmali agar was associated with generally higher uncertainty of measurement, and poor countability performance using plating volumes of 0.3 and 0.4 ml. CFA media performance was of questionable selectivity at naturally contaminated samples testing, and it was difficult to distinguish typical *Campylobacter* from non-*Campylobacter*, which might overestimate the real count.

Based on viewing results of the three levels of media testing in a context, the overall values of R, r, and U concluded by our study indicate that there are no major problems with the precision of ISO 10272-2:2006 protocol for *Campylobacter* enumeration based on spread plating using mCCDA medium. This protocol can be regarded as fit for the purpose of generating quantitative data on contamination of *Campylobacter* in poultry meat.

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